

Identification of a chromosomal gene controlling temperature-regulated expression of *Shigella* virulence

(virulence plasmid/HeLa cell invasion/operon fusion)

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ABSTRACT Genes required for the full expression of *Shigella* virulence are on both the chromosome and a large virulence-associated plasmid. Expression of one or more virulence (*vir*) genes is temperature-regulated, wild-type strains being virulent (invasive) when grown at 37°C but phenotypically avirulent (noninvasive) at 30°C. A *vir::lac* operon fusion located on the virulence plasmid, which brings the *lac* genes under control of a temperature-regulated *vir* gene promoter, was used to select regulatory mutants constitutive for the Lac⁺ phenotype at the nonpermissive temperature. A transposon Tn10-induced mutant that was Lac⁺ at 30°C and 37°C was isolated, and the Tn10 insertion was transduced into a wild-type strain. The transductants all simultaneously became deregulated for virulence and invaded HeLa cells equally well at 30°C and 37°C. Other virulence-associated phenotypes were also deregulated and expressed at 30°C. Southern hybridization with a probe for Tn10 determined the insertion to be on the chromosome. Fine mapping by transduction with phage P1L4 positioned the mutation between the *galU* and *trp* genes. A cosmid cloned fragment of *Shigella* chromosomal DNA containing the region around *galU* was used in complementation studies and showed that the closely linked regulatory gene was able to complement, in trans, the Tn10-induced mutation. We propose that this mutation defines a regulatory gene, *virR*, and that insertion of Tn10 into this gene inactivates a repressor that normally blocks expression of *vir* genes at 30°C.

The primary characteristic of *Shigella* pathogenicity is the ability of the bacteria to penetrate into and replicate within human colonic epithelial cells (1). Infection results in destruction of colonic epithelium and ulcerative lesions, producing the bloody diarrhea characteristic of bacillary dysentery. Laboratory methods for studying *Shigella* virulence use both animal and cell culture models for measuring penetration capacity and the ability to sustain growth inside mammalian cells. Use of these assays has permitted the identification of chromosomal virulence (*vir*) genes (2, 3) as well as the demonstration of the involvement of a 220-kilobase (kb) plasmid in expression of the virulence phenotype of *Shigella flexneri* (4). Large virulence-associated plasmids homologous to that found in *S. flexneri* are present in *Shigella sonnei*, *Shigella dysenteriae*, and enteroinvasive strains of *Escherichia coli* (5, 6). It also has been shown that expression of the invasive phenotype by *Shigella* spp. is regulated by growth temperature (7). Bacteria grown at 37°C are virulent and invade epithelial cells, whereas the same virulent strains are noninvasive when grown at 30°C. Loss of invasive ability at 30°C reflects a reversible phenotypic change and not a genotypic change, since virulence is completely restored after shifting the growth temperature back to 37°C.

We have taken advantage of gene fusion technology to study the mechanism of *vir* gene regulation. In a previous study, an operon fusion was constructed such that the structural gene for β -galactosidase (*lacZ*) was placed under the control of a temperature-regulated *vir* gene promoter, thereby providing evidence that this regulation is at the transcriptional level (8). In this paper, we describe the use of this *vir::lac* operon fusion to isolate mutants defective in the temperature regulation of *vir* gene expression. Mutants constitutive for expression of *vir* genes at 30°C and 37°C were isolated by selecting for constitutive expression of β -galactosidase in the mutant strain containing the *vir::lac* operon fusion. Analysis and mapping of one such mutant suggested that the temperature regulation of *vir* gene expression in *S. flexneri* is negatively controlled by a chromosomal gene. Complementation studies using a cosmid clone carrying the native chromosomal region to which the regulator gene mapped showed that the regulator behaved as a trans-acting repressor of *vir* gene expression.

MATERIALS AND METHODS

Bacterial Strains and Methods. All *S. flexneri* 2a strains are derivatives of the wild-type strain 2457T (9). BS184 is a phage λ -sensitive derivative of strain 2457T that contains a temperature-regulated *vir::lac* operon fusion in the virulence plasmid. This fusion, originally isolated with phage Mu d1(Ap^R *lac*) (ampicillin-resistant strain BS120 in ref. 8), was made temperature stable by recombination with Mu d11734, which carries a gene for kanamycin resistance (10). The resulting *vir::lac* operon fusion, which we call *vir-83::Mu d11734*, is heat stable, ampicillin sensitive, and kanamycin resistant. ATM016 (F⁻ *lacYl glnV tyrT galU trp srl recA56 metBI hsdR514 trpR55 λ*) is a derivative of *E. coli* K-12 constructed in this lab for the cloning experiments described in this study. Genetic manipulations, including transductional mapping with bacteriophage P1L4 (11), transformation (12), and transposon Tn10 mutagenesis with phage λ NK561 (13), were as described.

Media. Tryptic soy broth (Institut Pasteur Production, Marnes La Coquette, France) was used as the complete medium for growth of bacteria. Brain heart infusion agar (Difco) was used when preparing plates containing antibiotics. Selection for loss of tetracycline resistance (Tc^R) was done by plating bacteria for growth on fusaric acid plates (14). M9 salts (15) were used as the base for preparing minimal medium. Carbon sources were added to a final concentration of 0.5%, and the medium was supplemented with 10 μ g of nicotinic acid per ml for growth of *Shigella*.

Virulence Assays. The HeLa cell assay for invasion of epithelial cells by *Shigella* was performed as described (16).

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Abbreviations: Tc^R, tetracycline resistance; Tc^S, tetracycline sensitivity.

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Table 1. Expression of β -galactosidase from the *vir-83::Mu d11734* operon fusion

Strain	β -Galactosidase,* units		Ratio 37°C/30°C
	30°C	37°C	
BS184	6.5	167.0	25.7
BS185	108.0	280.0	2.6
BS186	101.5	241.0	2.4

* β -Galactosidase levels were measured by the method of Miller (15).

Other tests used to measure virulence were the Sereny test (17), the plaque assay of Oaks *et al.* (18), and the assay for contact-mediated hemolysis (19).

Hybridization Analysis. The transposon *Tn10* contains no sites for cleavage by *Sal* I or *Pst* I and is cut only once by *Eco*RI, 3.1 kb from one end of the 9.3-kb element (20). The 20.8-kb *Pst* I fragment of λ NK561, which contains *Tn10*, was nick-translated for use as a probe to localize *Tn10* insertions by Southern hybridization (21). Identification of virulence plasmid-associated peptides was accomplished by electrophoretic immunoblot hybridization as described (22).

Cosmid Cloning. High molecular weight *S. flexneri* chromosomal DNA was isolated from strain 2457T, and the DNA was partially digested with *Sau*3A (Boehringer Mannheim) to yield fragments in the size range of 20–30 kb. These fragments were ligated into the low-copy-number cosmid vector pCVD301 (23), which had been digested with *Bam*HI and treated with phosphatase. The ligated DNA was packaged into phage λ heads by using an *in vitro* packaging mix (Gigapack, Stratagene, San Diego, CA). Recombinant clones were isolated by selection for *Tc*^R with tetracycline at 5 μ g/ml.

RESULTS

Mutagenesis and Selection for Deregulated Expression of *vir-83::Mu d11734*. The strategy for isolating mutations in genes affecting thermoregulation of virulence was to mutagenize a strain carrying a *vir::lac* operon fusion and select for deregulation of the lactose-fermenting (*Lac*⁺) phenotype—i.e., expression of β -galactosidase at 30°C as well as 37°C. We chose transposon mutagenesis by *Tn10* because it creates stable insertions, permits selection by *Tc*^R, and allows one to select for loss of *Tc*^R by selection for resistance to fusaric acid (14).

Strain BS184 (*vir-83::Mu d11734*) was mutagenized with λ NK561 and *Lac*⁺ *Tc*^R transductants were selected at 30°C by plating on M9 lactose plates. Although the parental strain, BS184, does produce low detectable levels of β -galactosidase at 30°C, mutant colonies were found that grew much faster on M9 lactose plates at 30°C. Two of these mutants, BS185 and BS186, were purified, and levels of β -galactosidase production at 30°C and 37°C were measured. As shown in Table 1, both mutants were deregulated for expres-

sion of β -galactosidase at 30°C, indicating that they had mutations in a gene controlling the thermoregulation of expression from a *vir* gene promoter.

Characterization of Mutants for Temperature Control of *vir* Gene Expression. To confirm that the mutations in BS185 and BS186 altered thermoregulation of *vir* gene expression, the *Tn10* insertion mutation was moved into a wild-type (invasive) genetic background. P1L4 transducing lysates were prepared on the two mutants and used to transduce strain 2457T to the *Tc*^R phenotype. *Tc*^R transductants were first screened for virulence in the HeLa cell invasion assay. Whereas the parental strain 2457T was invasive when grown at 37°C but noninvasive after growth at 30°C, the transductants carrying the *Tn10* insertion (BS189 and BS190) were fully invasive in HeLa cells when grown at both 30°C and 37°C. This confirmed that the *Tn10* insertion isolated in BS184 created a defect that deregulated expression of the invasive phenotype in *S. flexneri*. A series of tests for expression of other temperature-regulated virulence phenotypes was performed on the mutants, and results are shown in Table 2. With the exception of pigmentation on Congo red medium, all of the temperature-regulated virulence properties tested were deregulated in the mutants and fully expressed at 30°C.

Immunoblot hybridization was used to test the mutant strains for expression of four temperature-regulated virulence-associated peptides encoded by the virulence plasmid. Both mutants expressed high amounts of the four peptides at both 30°C and 37°C, whereas the parent strain displayed temperature-regulated expression of the peptides (Fig. 1). Since the *Tn10* insertions in BS185 and BS186 resulted in the loss of temperature regulation of *vir* gene expression, we call the mutation *virR::Tn10*.

Mapping of *virR::Tn10*. To obtain a first approximation of the location of the *Tn10* insertions in BS185 and BS186, plasmid DNA and total genomic DNA were prepared for Southern blot analysis and hybridized with a DNA probe containing *Tn10* sequences. The results (Fig. 2) showed that the *Tn10* insertion was not in the virulence-associated plasmid but was chromosomal in both mutants, thus localizing *virR* to the chromosome. Hybridization of total cell DNA digested with *Sal* I yielded a single similar-size band in each mutant. Since *Sal* I does not cut within *Tn10* (20), this result suggested that only a single *Tn10* insertion existed in the mutants. Further support for this came from hybridization with the *Tn10* probe of *Eco*RI-digested total cell DNA. *Eco*RI cuts once within *Tn10*. Therefore, a single *Tn10* insertion in the mutant strain would be expected to yield two bands hybridizing with the probe. That is the case with the two mutant strains shown in Fig. 2. The *Sal* I and *Eco*RI fragments from each mutant are of similar sizes, so it is likely that the insertions are in the same region in both BS185 and BS186.

To further map the *Tn10* insertions, we exploited the fact that imprecise excision of *Tn10* occurs at a detectable frequency and often generates deletions adjacent to the site

Table 2. Expression of virulence-associated phenotype in wild-type and mutant strains of *S. flexneri* 2a

Strain	t, °C	HeLa cell invasion,* %	Sereny test	Contact hemolysis†	Pigmentation on Congo red‡	Plaque assay
2457T	30°C	<1	—	0.033	—	—
	37°C	95	++	2.18	+++	+++
BS189	30°C	89	++	1.96	—	+++
	37°C	94	++	2.17	+++	+++
BS190	30°C	72	++	1.90	—	+++
	37°C	86	++	2.00	+++	+++

*Percentage of infected HeLa cells.

†Activity is expressed as OD₅₄₅ as described by Sansonetti *et al.* (19).

‡Pigmentation was tested on tryptic soy broth plates containing 1.5% agar and 0.006% Congo red.

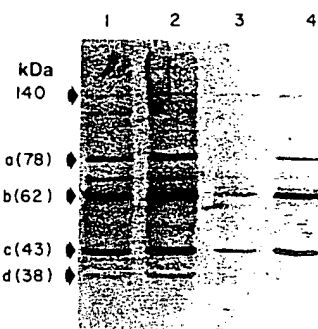


FIG. 1. Immunoblot analysis for expression of virulence-associated peptides, showing a blot of polyacrylamide gel containing total bacterial protein from BS189 grown at 30°C (lane 1) and 37°C (lane 2) and wild-type *S. flexneri* grown at 30°C (lane 3) and 37°C (lane 4). Midlogarithmic-phase bacterial cultures were harvested and adjusted to identical OD₆₀₀ units before lysing the cells and loading the gels. The blotted gel was treated with convalescent serum from a monkey infected with *S. flexneri*, and bound antibody was detected with alkaline phosphatase-conjugated protein A. This serum specifically recognizes four major virulence plasmid-encoded peptides, a-d (24), and these are identified by the arrows.

of insertion (25). Loss of *Tn10* results in tetracycline sensitivity (Tc^S), and these mutants can be selected by their resistance to fusaric acid (14). We reasoned that if *virR::Tn10* was located close to a gene involved in biosynthesis of an essential metabolite, one should find auxotrophic mutants among the fusaric acid-resistant derivatives because of *Tn10*-promoted excision/deletion extending into the adjacent gene. In three separate experiments, at least 100 fusaric acid-resistant derivatives of BS185 were selected and screened for acquisition of an auxotrophic requirement. Four independent auxotrophs were isolated, and all four were found to have picked up a requirement for tryptophan. This result placed the *Tn10* insertion near the *trp* operon. Because of the close proximity of the *galU* gene to *trp*, we also tested these *Tn10* excision deletion mutants for galactose sensitivity, a phenotype of *galU* mutants. All four *trp* auxotrophs were galactose resistant and therefore GalU⁺. These $\Delta(virR-trp)$ deletion mutants and several Trp⁺ fusaric acid-resistant derivatives tested retained the deregulated phenotype of the parental *virR::Tn10* mutation.

Fine mapping by P1L4 transduction further defined the chromosomal location of *virR*. In transductional crosses with an isogenic *trp* recipient, *virR::Tn10* showed 63%

linkage with *trp*. Similar crosses with a recipient carrying a deletion in *galU* showed 100% linkage of *virR* with GalU⁺. The transductional linkage between *galU* and *trp* in *E. coli* K-12 is about 50% (26), and we have found the linkage in *Shigella* to be between 50% and 60% (unpublished results). The transductional mapping data are consistent with a gene order of *galU-virR-trp*. Further evidence of this gene order came from fusaric acid-resistant mutants of BS185 (see above), which became auxotrophic for tryptophan but remained GalU⁺. This indicated that the deletion extending from *virR* into *trp* did not include *galU*.

Cloning of the Native *virR* Gene and Complementation in Trans of the *virR* Mutation. A cosmid clone bank of total DNA extracted from strain 2457T was screened for the wild-type *virR* gene by first selecting for the closely linked marker *galU*. ATM016, a *galU trp recA* derivative of *E. coli* K-12, was infected with the cosmid lysate, and Gal⁻ Tc^R recombinants were selected. Four clones that complemented *galU* were checked for complementation of the *trp* defect to determine whether any of the clones included the region *galU-virR-trp*. All four, however, were Trp⁻. This was not unexpected because the maximal-size fragment clonable in the vector used, pCVD301, would barely be large enough to contain this interval. Plasmid DNA was isolated from one clone (pATM003) and transformed into BS255 (a *recA*, Tc^S derivative of BS185) to score for the presence of the wild-type *virR* gene and complementation of the *virR* mutation. Transformants were tested for β -galactosidase levels after growth at 30°C and 37°C. Clone pATM003 restored temperature-regulated expression of the *vir-83::Mu d11734* operon fusion in BS255. Levels of β -galactosidase expression in BS255 carrying pATM003 showed a decrease by a factor of >10 after growth at 30°C as compared to the strain without the clone. Expression at 37°C was still higher than at 30°C, though the total levels were less than in the deregulated mutant. This demonstrated in trans complementation of the *virR* mutation by a gene(s) on pATM003.

DISCUSSION

The expression of *Shigella* virulence is regulated by growth temperature, and a variety of virulence-associated phenotypes is affected. Studies on the genetics of *Shigella* virulence have shown that chromosomal (2, 3) as well as noncontiguous plasmid-encoded genes (27) are necessary for virulence. Since *Shigella* virulence is multigenic and responds to a common signal, temperature, it is likely that the *vir* genes themselves comprise a virulence regulon—a net-

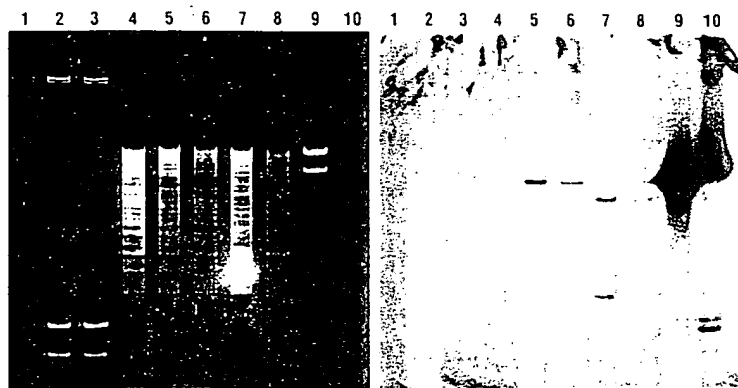


FIG. 2. Localization of *Tn10* insertions in mutants BS185 and BS186. (Left) Ethidium bromide-stained 0.7% agarose gel of plasmid and total DNA from *S. flexneri*. Lanes: 1-3, plasmid DNA from 2457T (lane 1), BS189 (lane 2), and BS190 (lane 3); 4, total DNA from 2457T digested with *Sal* I; 5 and 7, total DNA from BS189 digested with *Sal* I (lane 5) and *Eco*RI (lane 7); 6 and 8, total DNA from BS190 digested with *Sal* I (lane 6) and *Eco*RI (lane 8); 9, phage λ DNA digested with *Sal* I; 10, λ NK561 DNA digested with *Pst* I. (Right) Autoradiogram of the gel in Left blotted and hybridized with the 20-kb *Pst* I fragment of λ NK561, which contains *Tn10*.

work of diverse unlinked genes that share a common regulatory signal. The experiments described in this paper were designed to exploit a *vir::lac* operon fusion to examine the mechanism underlying the temperature control of this regulon.

The Tn10 insertion mutation described here deregulates the expression of the operon fusion at 30°C, resulting in constitutive expression from the fused *vir* gene promoter. This strongly suggested that the insertion was in a regulatory gene. Transduction of the mutation into a wild-type strain deregulated expression of a variety of virulence phenotypes, confirming that the Tn10 had caused a mutation that affected the thermoregulation of *vir* gene expression and was not a mutation that only altered expression of the *lac* operon genes in the original fusion. In the constitutive mutants, β -galactosidase expression, though now derepressed, was still slightly higher at 37°C than at 30°C. One explanation for this may be an overall higher level of cellular metabolism of the bacteria at the higher temperature. On the other hand, additional as-yet-undefined regulatory genes may play a role in controlling the expression of these genes.

Congo red binding is a phenotype that is tightly associated with virulence of *Shigella* spp. (28) and also is temperature-regulated (7). The *virR* mutant, however, did not constitutively express Congo red binding as expected. One possible interpretation of this is that the gene product responsible for Congo red binding has dual but separable phenotypes. In this case, perhaps the gene product is produced in the mutant at 30°C but is not functional at this temperature in its Congo red binding ability. This does not preclude its still retaining the function important in expression of virulence. Another possibility is that, although closely associated with the virulence phenotype, Congo red binding is not encoded by a *vir* gene and, therefore, is not part of the *vir* regulon.

In the course of these studies, we also measured the effect of temperature on production by *S. flexneri* of aerobactin (29) and Shiga-like toxin (30), both of which are thought to play roles in virulence. Aerobactin production was not temperature-regulated for expression (data not shown). This is consistent with recent data from two laboratories that indicate that mutants of *S. flexneri* defective in aerobactin-mediated iron transport are only slightly altered in their virulence properties (31, 32). This suggests that aerobactin gene expression is not a part of a *vir* regulon. Shiga toxin production, however, seems to be regulated by growth temperature (33).

Since insertion of Tn10 into a gene usually causes inactivation of the gene (25), we postulated that the Tn10 insertion inactivated a gene that normally encodes a repressor of *vir* gene expression. Abolishing expression of the repressor would result in constitutive expression of *vir* genes. Insertion of Tn10 also is known to sometimes turn on transcription of adjacent chromosomal genes by means of a promoter carried in the flanking insertion sequence IS10 elements of the transposon (34). Activation of a regulatory gene by Tn10 insertion could also be an explanation for the results observed here. Evidence against this alternative was provided by the observation that all spontaneous fusaric acid-resistant derivatives of the *virR::Tn10* mutation still expressed the deregulated phenotype. Since the majority of fusaric acid-resistant derivatives of Tn10 result from imprecise excision of the transposon accompanied by deletion formation and gene rearrangement (25), it is unlikely that insertional activation of an adjacent gene would be preserved in all such mutants. Therefore, the most likely model was that the Tn10 insertion inactivated a repressor of *vir* gene expression.

The operon fusion used to select for regulatory mutations was located on the virulence plasmid, yet the mutation isolated, *virR*, mapped to the chromosome. This indicated that the gene product of *virR* must act in trans. Confirmation

of this model came from results of in-trans complementation of the *virR* defect with a cloned wild-type *virR* gene. The cloned *virR* gene restored temperature regulation of *vir* genes in the mutant strain, thus demonstrating that the *virR* gene product is a trans-acting substance that represses *vir* gene expression at 30°C while permitting expression of the *vir* regulon at 37°C. Considering the fact that the genes involved in *Shigella* virulence are located on both the chromosome and a plasmid, a diffusible trans-acting regulator, such as a repressor, would be an efficient means of global regulation of these noncontiguous genes.

The high-temperature regulon (heat shock response) of *E. coli* is another example of global control that is triggered by growth temperature (35). Temperature regulation of *vir* gene expression in *Shigella* is distinct from this system for several reasons. In the heat shock response of *E. coli*, expression of the regulon is stimulated by a shift in temperature above 37°C. Thermal shock results in a transient expression of the genes in the regulon. Thus, the heat shock response appears to be a cellular reaction to environmental stress. Induction of the *vir* regulon in *Shigella* involves a temperature shift over the range for normal growth of the bacteria and its mammalian host, and activation of the regulon does not appear to be transient. Nevertheless, it would be interesting to compare the control mechanisms of these two regulons and the structures of the regulatory gene products to get a better understanding of how bacteria can "sense" a change in temperature.

Temperature regulation of *vir* gene expression may prove to be a universally used mechanism of gene regulation in pathogenic bacteria. *Vir* gene expression responsive to growth temperature has been observed in other pathogens including *E. coli* [pili and colonization factors (36, 37)], *Yersinia enterocolitica* [serum resistance (38)], *Y. pestis* [virulence-associated proteins (39, 40)], and *Bordetella pertussis* [modulation of expression of phenotypic markers associated with virulence (41)]. The system we have described in *Shigella* can thus serve as a model for studying such regulatory phenomena in other pathogens.

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